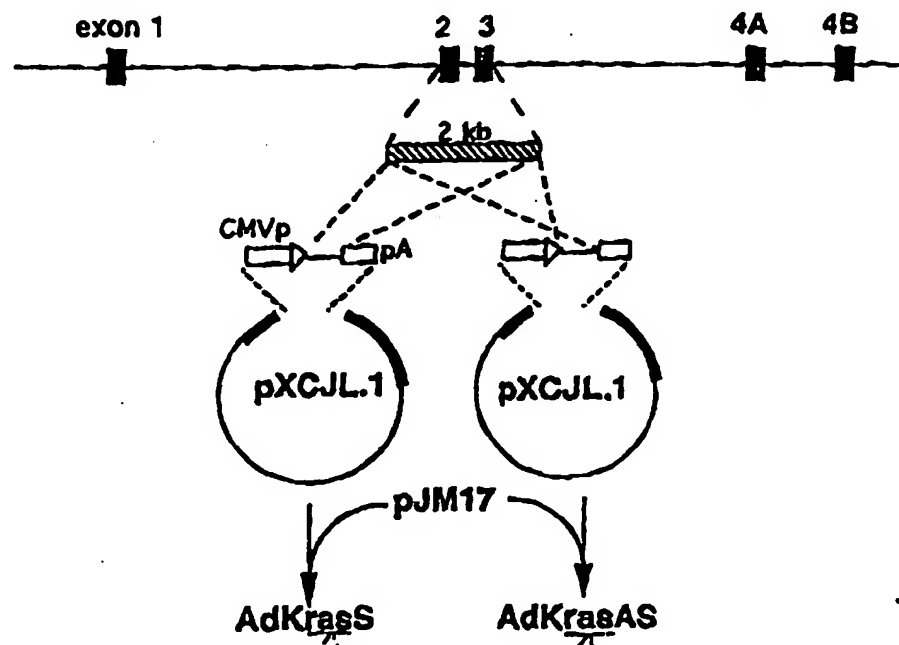




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(54) Title: ADENOVIRUS-ANTISENSE K-ras EXPRESSION VECTORS AND THEIR APPLICATION IN CANCER THERAPY



(57) Abstract

A variety of genetic constructs are disclosed that will find both *in vitro* and *in vivo* use in the area of tumor biology and cancer therapy. In particular, adenoviral expression vectors are provided that contain a K-ras nucleic acid positioned antisense to regulatory control regions. In one embodiment, the adenoviral expression vector is a replication-deficient adenoviral vector lacking the E1 region and containing a K-ras nucleic acid. Also provided are methods for the inhibition of cancer cell proliferation.

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DESCRIPTIONADENOVIRUS-ANTISENSE K-ras EXPRESSION VECTORS
AND THEIR APPLICATION IN CANCER THERAPY

5

BACKGROUND OF THE INVENTION1. Field of the Invention

10 The present invention relates generally to the field
of tumor biology. In particular, the invention relates
to a polynucleotide encoding an antisense construct that
targets a known oncogene. In one embodiment, the
invention relates to adenovirus expression vectors
15 encoding an antisense K-ras and their use in inhibiting
cancer.

2. Description of the Related Art

20 Cancer is one of the leading causes of human
disease, being responsible for 526,000 deaths in the
United States each year. Lung cancer alone kills more
than 140,000 people annually in the United States.
Recently, age-adjusted mortality from lung cancer has
25 surpassed that from breast cancer in women. Although
implementation of smoking-reduction programs has
decreased the prevalence of smoking, lung cancer
mortality rates will remain high well into the
twenty-first century. Unfortunately, current treatment
30 methods for cancer, including radiation therapy, surgery
and chemotherapy, are known to have limited
effectiveness. The rational development of new therapies
for lung cancer largely will depend on gaining an
improved understanding of the biology of cancer at the
35 molecular level.

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With advances in molecular genetics and biology, it has become evident that altered expression of normal genes can lead to the initiation of transforming events that result in the creation of cancer cells. The
5 conventional therapy for malignancy, such as chemotherapy and radiation, has focused on mass cell killing without specific targeting, often resulting in damaging side effects. A new direction in cancer therapy is to deliver
10 a normal gene to replace or correct the mutated gene, thereby altering the malignant phenotype of transformed cells. Several expression vectors have been developed in order to deliver a gene into somatic cells with high efficiency.

15 The *ras* gene family is, perhaps, the best characterized of the oncogene families. Most of the identified transforming genes in human carcinomas have been members of the *ras* family, which encode immunologically related proteins that have a molecular
20 weight of 21,000 (designated p21). Nucleotide sequence analysis of several *ras* mutants reveals different mutations, but amino acid residues 12 and 61 appear to play an important role. Tabin (1982); Der and Cooper (1983); Yuasa et al. (1983).

25 Mutations of the *ras* gene family are found in more than 30% of human carcinomas, especially those of pancreas, colon and lung. The role of these mutations in tumorigenesis, invasion and metastasis has been well
30 documented. Barbacid (1985); Bos (1989). Protein products of mutated *ras* genes permanently transduce a strong mitogenic signal to stimulate cell proliferation. Therefore, blocking mutated *ras* has a clear antitumor potential, and different strategies have been used to
35 achieve this objective. For example, the neoplastic phenotype associated with mutated *ras* genes has been reversed by antibodies to p21, by fragments of natural

- 3 -

p21 ligands (e.g., NF1 and c-Raf-1), and by dominant negative *ras* mutants. Mulcahy et al. (1985); Fridman et al. (1994); Ogiso et al. (1994). At the mRNA level, strategies have been based on anti-H-*ras* ribozymes and antisense oligonucleotides. Kashani-Sabet et al. (1992); Brown et al. (1989); Debus et al. (1990). In addition, targeting of DNA has been accomplished by homologous recombination. Shirasawa et al. (1993).

Up to 30% of all lung adenocarcinomas have a mutated *ras* gene, and more than 90% of these mutations occur in the K-*ras* gene. In the neoplastic development of lung tumors, K-*ras* mutations may arise prior to invasion and can easily be detected in sputum samples. The presence of this mutation correlates with a poor clinical outcome. Initial studies have shown that K-*ras* expression in tumor cell lines can be inhibited by transfection of a plasmid construct that expresses a K-*ras* antisense RNA. This K-*ras* construct was then inserted into a retroviral vector and similar results were achieved following infection of tumor cells and in an orthotopic nude mouse model. Mukhopadhyay et al. (1991); Georges et al. (1993). Despite these results, the retroviral system is not without its limitations. For example, vector-borne genotoxicity is associated with integration. Retroviruses also are unstable, require specific receptors for entry in to cells and replicate only in actively proliferating cells. Thus, there remains a need for improved gene therapeutic compositions for use in anticancer treatments.

3. Summary of the Invention

The present invention addresses the need for improved therapy for lung and other K-*ras*-associated cancers by providing adenoviral expression vectors containing a polynucleotide encoding a K-*ras* antisense

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transcript. It also is an object of the present invention to provide methods for the use of such compositions and, in particular, use in the treatment of cancer.

5

The present invention encompasses adenovirus expression vectors that comprise a promoter functional in eukaryotic cells and a polynucleotide encoding a K-ras antisense construct, the polynucleotide being under
10 transcriptional control of the promoter and positioned such that the transcript produced is antisense. In a preferred embodiment, the adenovirus lacks at least a portion of the E1 region. In another embodiment, the adenoviral expression vectors further comprise a
15 polyadenylation signal. In yet another embodiment, the constructs further comprise a selectable marker.

In certain embodiments, the polynucleotide is derived from the genome. In other embodiments, the
20 polynucleotide is a cDNA or synthetically generated polynucleotide. Still other embodiments include a combination of cDNA and genomic DNA, for example, in a mini-gene construct. Further embodiments include fragments of K-ras that correspond to introns and/or
25 splice junctions.

The present invention also includes pharmaceutical compositions comprising an expression vector with a promoter functional in eukaryotic cells and a
30 polynucleotide encoding a K-ras antisense transcript, along with a pharmaceutically acceptable buffer, solvent or diluent. In certain embodiments, the expression vector and pharmaceutically acceptable buffer, solvent or diluent are supplied in a kit.

35

The present invention further comprises a method for inhibiting K-ras function in a cell. This method

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comprises contacting such a cell with an expression vector as described above, wherein the polynucleotide is positioned in an antisense orientation with respect to the promoter. In an exemplary embodiment of the invention, the cell is a transformed cell and the contacting reverses the transformed phenotype. In a further embodiment, the cell is a lung, pancreas or colon cancer cell.

Another embodiment of the invention is a method of treating a mammal with cancer. This method comprises administering to an animal a pharmaceutical composition comprising an expression vector having a promoter functional in eukaryotic cells and a polynucleotide encoding a K-ras antisense transcript, in a pharmaceutically acceptable buffer, solvent or diluent. In a particular embodiment of the invention, the mammal is a human. In another embodiment, administering is via intratumoral instillation. In a further embodiment, the cancer is lung cancer.

4. Brief Description of the Drawings

FIG. 1 - Adenoviral Vector Construction. A 2 kB genomic fragment containing exons 2 and 3 and intron 2 of the K-ras protooncogene was cloned between the CMV promoter and the SV40 polyadenylation signal in sense and antisense orientations. These expression constructs were inserted into the polylinker site of pXCJL.1, which contains the left arm of Adenovirus type 5 (Ad5) with the exception of an E1 deletion. These plasmids were individually cotransfected into 293 cells with pJM17, a non-packageable Ad5 genome, which results in rescue of the constructs into the Ad5 genome and renders the recombinant packageable.

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FIG. 2 - Growth Curve of Transduced H460a Cell In Vitro. At the indicated days following initial infection (MOI of 100 pfu/cell, day 0), cells were incubated with [³H]thymidine for 4 h and harvested, and the incorporated radioactivity was counted (cpm). The plot represents combined data from three studies. Similar curves were obtained by cell counting (P<.001) by analysis of variance test.

5. Detailed Description of the Preferred Embodiments

Previous studies have shown that regions of the K-ras gene can be used as antisense constructs to inhibit the expression of the K-ras product and, in so doing, reverse the transformed phenotype of tumor cells in which the K-ras product is aberrant, either in level of expression or in sequence. This has been accomplished by using both expression plasmids in vitro and retroviral vectors in vivo. A particular region of the K-ras gene was used, spanning exons 2 and 3 and including the intervening intron 2, which allowed discrimination between the oncogene and other ras-related sequences.

Here, those studies are extended to the use of a genetically engineered adenovirus expression vector. An adenoviral vector carrying a 2 kB fragment of the K-ras protooncogene, inserted in an antisense orientation to the construct promoter, was used to infect H460a lung cancer cells. Efficient transfer and high level expression from the construct were observed. At a multiplicity of infection of 100, 65% of cells were transduced and K-ras production was reduced by 70%. This resulted in a 40% inhibition of monolayer growth and, interestingly, a 90% inhibition of colony formation.

Thus, the present invention involves the use of adenoviral expression vectors in the reversal of the

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transformed state of certain tumor cells. The adenovirus genome provides an advantageous framework in which to insert a therapeutic gene, in this instance, an antisense polynucleotide for a K-ras antisense construct.

- 5 Preferred forms of the virus are replication defective and can only be grown on special, helper cell lines that provide the missing replicative functions in trans. Such an engineered adenovirus can be propagated in vitro to high titers for use in treating cancer cells.

10

- It is proposed that antisense constructs containing introns bind to "sense" intron regions found on the RNA transcript of the gene, and affect proper RNA processing. Thus, subsequent translation of protein-coding RNA's into their corresponding proteins is inhibited or prevented.
- 15 The use of antisense introns may prove advantageous, in certain situations, because genetic diversity in non-coding regions may be higher than in coding regions.

20

- As used herein, the term "intron" is intended to refer to gene regions that are transcribed into RNA molecules, but processed out of the RNA before it is translated into a protein. In contrast, "exon" regions are those which are translated into protein.

25

- Thus, where one seeks to selectively inhibit a particular gene or genes over a related gene, as is the case with ras genes, one embodiment proposes to target distinct intron regions. A "distinct" intron region, as used herein, is intended to refer to an intron region that is sufficiently different from an intron region of another gene such that cross hybridization would not occur under physiologic conditions. The intracellular concentration of monovalent cation is approximately 160 mM (10 mM Na⁺; 150 mM K⁺). The intracellular concentration of divalent cation is approximately 20 mM (18 mM Mg⁺; 2 mM Ca⁺⁺). The intracellular protein
- 30
- 35

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concentration, which would serve to decrease the volume of hybridization and, therefore, increase the effective concentration of nucleic acid species, is 150 mg/ml.

Constructs can be tested in vitro under conditions that
5 mimic these in vivo conditions. Typically, where one intron exhibits sequence homology of no more than 20% with respect to a second intron, one would not expect hybridization to occur between antisense and sense introns under physiologic conditions.

10

The following description defines the invention in detail.

**A. K-ras AND K-ras-RELATED ANTISENSE
POLYNUCLEOTIDES**

15

The term "K-ras antisense polynucleotide" is intended to refer to molecules complementary to the RNA of K-ras or the DNA corresponding thereto.

20 "Complementary" polynucleotides are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C)
25 and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with
30 pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation.
35 Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing,

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transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs will include regions complementary to intron/exon splice junctions. Thus, it is proposed that a preferred embodiment includes an antisense construct with complementarity to regions within 50-200 bases of a intron/exon splice junction. It has been observed that some exon sequences can be included in the construct without seriously affecting the target selectivity thereof. The amount of exonic material included will vary depending on the particular exon and intron sequences used. One can readily test whether too much exon DNA is included simply by testing the constructs *in vitro* to determine whether normal cellular function is affected or whether the expression of related genes having complementary sequences is affected.

25

As used herein, the terms "complementary" or "antisense sequences" mean polynucleotide sequences that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have a complementary nucleotide at thirteen or fourteen positions. Naturally, sequences which are "completely complementary" will be sequences which are entirely complementary throughout their entire length and have no base mismatches.

35

- 10 -

Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (e.g., a ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

The polynucleotides according to the present invention may encode an entire K-ras gene or a portion of K-ras that is sufficient to effect antisense inhibition of ras expression. The polynucleotides may be derived from genomic DNA, i.e., cloned directly from the genome of a particular organism. In other embodiments, however, the polynucleotides may be complementary DNA (cDNA). cDNA is DNA prepared using messenger RNA (mRNA) as template. Thus, a cDNA does not contain any interrupted coding sequences and usually contains almost exclusively the coding region(s) for the corresponding protein. In other embodiments, the antisense polynucleotide may be produced synthetically.

It may be advantageous to combine portions of the genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

The DNA and protein sequences for K-ras are provided below. It is contemplated that natural variants of K-ras exist that have different sequences than those disclosed herein. Thus, the present invention is not limited to use of the provided polynucleotide sequence for K-ras but, rather, includes use of any naturally-occurring

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variants. Depending on the particular sequence of such variants, they may provide additional advantages in terms of target selectivity, i.e., avoid unwanted antisense inhibition of K-ras-related transcripts. The present
5 invention also encompasses chemically synthesized mutants of these sequences.

Another kind of sequence variant results from codon variation. Because there are several codons for most of
10 the 20 normal amino acids, many different DNA's can encode the K-ras shown in FIG. 4. Reference to the following table will allow such variants to be identified.

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TABLE 1

<u>Amino Acids</u>			<u>Codons</u>						
	Alanine	Ala	A	GCA	GCC	GCG	GCU		
5	Cysteine	Cys	C	UGC	UGU				
	Aspartic acid	Asp	D	GAC	GAU				
	Glutamic acid	Glu	E	GAA	GAG				
	Phenylalanine	Phe	F	UUC	UUU				
	Glycine	Gly	G	GGA	GGC	GGG	GGU		
10	Histidine	His	H	CAC	CAU				
	Isoleucine	Ile	I	AUA	AUC	AUU			
	Lysine	Lys	K	AAA	AAG				
	Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU
	Methionine	Met	M	AUG					
15	Asparagine	Asn	N	AAC	AAU				
	Proline	Pro	P	CCA	CCC	CCG	CCU		
	Glutamine	Gln	Q	CAA	CAG				
	Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU
	Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU
20	Threonine	Thr	T	ACA	ACC	ACG	ACU		
	Valine	Val	V	GUA	GUC	GUG	GUU		
	Tryptophan	Trp	W	UGG					
	Tyrosine	Tyr	Y	UAC	UAU				

25

. Allowing for the degeneracy of the genetic code,
 sequences that have between about 50% and about 75%, or
 between about 76% and about 99% of nucleotides that are
 identical to the nucleotides disclosed herein will be
 30 preferred. Sequences that are within the scope of "a
 K-ras antisense polynucleotide" are those that are
 capable of base-pairing with a polynucleotide segment

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containing the complement of the K-ras sequences disclosed herein as SEQ ID NO:1 through SEQ ID NO:7 under intracellular conditions.

5 As stated above, although the K-ras antisense sequences may be full length genomic or cDNA copies, or large fragments thereof, they also may be shorter oligonucleotides. Sequences of 17 bases long should
10 suffice to specify a unique target sequence. Although shorter oligomers are easier to make and increase in vivo accessibility, numerous other factors are involved in determining the specificity of base-pairing. Both
15 binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 base pairs will be used.

20 While all or part of the gene sequence may be employed in the context of antisense construction, statistically, any sequence of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence. Although shorter
25 oligomers are easier to make and increase in vivo accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases
30 with increasing length. It is contemplated that oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more base pairs will be used. One can readily determine whether a given antisense polynucleotide is effective at targeting of the
35 corresponding host cell gene simply by testing the constructs in vitro to determine whether the endogenous

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gene's function is affected or whether the expression of related genes having complementary sequences is affected.

In certain embodiments, one may wish to employ antisense constructs which include other elements, for example, those which include C-5 propyne pyrimidines. Oligonucleotides which contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression (Wagner et al., 1993).

As an alternative to targeted antisense delivery, targeted ribozymes may be used. The term "ribozyme" is refers to an RNA-based enzyme capable of targeting and cleaving particular base sequences in K-ras DNA and RNA. Ribozymes can either be targeted directly to cells, in the form of RNA oligonucleotides incorporating ribozyme sequences, or introduced into the cell as an expression vector encoding the desired ribozymal RNA. Ribozymes may be used and applied in much the same way as described for antisense polynucleotide. Ribozyme sequences also may be modified in much the same way as described for antisense polynucleotide. For example, one could incorporate non-Watson-Crick bases, or make mixed RNA/DNA oligonucleotides, or modify the phosphodiester backbone.

The nucleotide and amino acid sequences of K-ras are as follows:

The following sequence includes a genomic fragment of K-ras from base 67 to base 1961. This genomic fragment includes exon 2. The exon begins at base 618 and ends at base 796. The underlined sequences are examples of oligonucleotide primer hybridization sequences to be used in the practice of the present invention.

-15-

ATGCAGAGGC CGAGGCCGCC TCGGCCCTCTG AGCTATTCCA GAAGTAGTGA GGAGGCTTTT
TTGGAGGTAT GAACAGACAC TTAAGTTTATT TCCACATTTG GGGTATTATA AATAGTGTCTG
CTGCGAACAT TGGTGACAT GTATCTGTTT GAGTCCCTGT NNTTAGTTAT TTTGGTTATA
TACCTAGGAA TGGAAATNGCT GATCATATGG TAATTCTGTG TTTAACTTTN TGAGGAACATA
CCACTGTTTT CCACAATGGC ATCACCCTATT TTACATTCCC ACCAGCAATG CACAAAGATT
TCAGTGTCTG TATCCTTGT AACTTTATT TTCCATTTN TGAGTTTNA TGTTTTGGTC
TNNTAATAA TAGCCAATCC TAATGGGTAT GTGGTAGCAT CTCATGGCTT GGATTTTATT
TTCCTGACTA TTGATGATGT TGAGCATCTT TTCAGGTGCT TAGTGGCCAT TTGTCCGTCA
TCTTTGGAGC AGGAACAATG TCTTCAAGTC CTTTGCCCAT TTTTAAATTG AATTTTGT
TGTGAGTTG TATATAACAC CTTTTTTGAA GTAAAAGGTG CACTGTAATA ATCCAGACTG
TGTTTCTCCC TTCTCAGGAT TCCTACAGGA AGCAAGTAGT AATTGATGA GAAACCTGTC
TCTTGGATAT TCTCGACACA GCAGGTCAAG AGGAGTACAG TGCAATGAGG GACCAGTACA
TGAGGACTGG GGAGGCTTT CTTTGTTGAT TTGCCATAAA TAATACTAAA TCATTTGAAG
ATATTCACCA TTATAGGTGG GTTTAAATTG AATATAATAA GCTGACATTA AGGAGTAATT
ATAGTTTTTA TTTTGTGAGT CTTTGCTAAT GCCATGCATA TAATATTTAA TAAAAATTTT
TAAATAATGT TTATGAGGTA GGTAAATATCC CTGTTTTATA AATGAAGTTC TTGGGGGATT
AGAGCAGTGG AGTAACTTGC TCCAGACTGC ATCGGTAGTG GTGGTGTCTGG GATTGAAACC
TAGGCCTGTT TGACTCCACA GCCTTCTGTA CTCCTGACTA TTCTACAAA GCAAGACTTT
AAACTTTTTA GATACATCAT TAAAAAGAA AACCATAAA AAGAATATGA AAAGATGATN
TGAGATGGTG TCACTNTAAC AGTCTTANAN ACAATCGNGT GTATAGCATA GAATGCTGGA
TTGGATAAAC CAGTGGCAAT AAAAATTTT AAAGAATAAA AGTTTTGAAA GATTGAAGAA
GTTGGGCATT ACAGTTCTCT TAAATCTGAC CAAGCTGCAT AAAACCTATT AAAATAATCA
TTATTATGCT ATTTTATATT CTATTTCTTT GAGGGTTTAG TTTTCCCCCA AACTACATAT

5

10

15

20

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TAAGCAAATG AATCACTCAG TGGCTATGTC ATATAATAAC GAGTTAGCCT AGTTATAAGA
AGTTTAACAT TTTATTTAAG AACATTGTTA CAGCATGTTT ACTGTATAGT CTAGTAATAG
AGGAAAGAC ATTTGGGTGG GTGGTAGTGG TAGTATTTTT ATAGAGGAGT TACCAAATTT
CAGCTCTATT ATCCAAGTTT ACCCAGCTAA TGGTGTTCGG AACCGGAAT TTGAGCCAAT
5 TGTGACTCTG TTGGCTGCTC TGCTCCTTCT TTTGTGCTGT GTCTTTGAAA AGTCACCTAA
AATTGTGAGG GAATGTAATT TCACCCCAA TTTAGAGTTT ATGCACTTGT TATATTGAAA
ATGATTAAAC TGTAGAAGGG CTTTAAATGG AATAAGTGGT GTAGTAACTT CAGTGTGCCC
TACCTAGAAA GGAAAATCTT TCTAGTTGTC CACTTTGTTT TTTGAAAAAG TAATATGAAA
ATTATGTTAA TGCTTTAATT CAGGTTTTTG TAAAATATTT TTTATCTTTA CACATTTAAC
10 ATACGTTTCT AAAATTATAG TCTGTTATAT AGCACTTTGG GTAGATCCAG CTTGGGCTGC
AGGTCGACTC TAGCTGGAG AATAGCCGGG CGCGCTGTGA GCGGAAGTCG CCCC CGCCCT
GGCCACTTCC GCGCGCCGA GTCCTTAGGC CGCTAGGGG CGCCGGCGCG CGCAGCATTG
GGGATAAAGG AAGCCGGCC GCGCGTTAT TACCATAAAA GGCAACACT GGTGCGAGGC
GTCCCCGCGG CGCGCGGCAG GAAGCCAGGC CCAACCCCC TCCCAACCGG GCGCCAGCCC
15 CGCCTCGCC CGRTTCAAAC AGCGCCGGGT CGCGCGCGCG CACGCAGCGG CCACACCCCTC
GGCGGGCCRG CGGCTCGGC AGGAAGTGG CAAGCGCCCG GGAGCGCGCG CGACCCACC
CCTTCCGACC GAGCCGCCT TCGCCCRGC CCRGGCCGCG GCACCCCGG CCCCAGAACG
CAGCCGCAAT TAGCGCCTTG AGTCCCRGGC CGCAGCCGCA ATTAGCGCA ATTCCCRGCG
CGCAGCAGT TAGGCCCCAA AGGACCAGCG CGCAGCGCA TGGGCCCCCA GCCCCACCG
20 GGCCTGGCG GGGCTACGC CGGCCACCC TCGGATCCCC ATTGGCAAG GCGCGCTCA
GACAAAGACC CGCGCGTTG CCCCCGCCG GAGAGCGCA CCCC GGCGTCCCG
AGCGGGCCT CGCGCCTCG AACTGGCGTG GGGTGTCCC CATCTCCGA GGCCAGGGG
CTTCTCCGC GCCCCCAGC GCGGTCCGGT TCCCCCCCC TGCGCCCCC GCTGCGGCC

AGACGGCGGC TCTGCACGGG CGAAGGGGCC GGGCGCGCAT GCCCGGTG GCTGGCCGGG
CTTACCTGGC GCGGGGTGTG GACGGGCGGC GGATCGGCAA AGCGAGGCT CTGTGCTCGC
GGGCGGACG CGGTCTCGGC GGTGGTGGC CGTCGCGCCG CTGGGTTTA TAGGGCGCCG
CCGCGCGCGC TCGAGCCATA AAAGGCAACT TTCGGAACGG CGCACGCGAT TGGCCCCCGC
5 CCGCTCACTC ACCGGCTTCG CCGACAGTGC AGCATTTTTT TACCCCTCT CCCCTCCTTT
TGCGAAAAA AAAAAGAGCG AGAGCGAGNA TTGAGGAAGA GGAGGAGGA GAGTTTGGC
GTTGGCCGCC TTGGGGTGCT GGGCCCGGG GCTGGGGGCG CGCGCCGTGG CCCCCCGCC
CCAGCTGGG CAGTGCCCGG TTGGGCCCG CATGGCCAGG CCGCCCCCG GCCTGCCCGT, SEQ ID NO:1.

10 The following sequences are genomic sequences from GenBank accession numbers L00045,
K000652 and K000653.

-17-
The first sequence includes exon 1. The coding sequence is from base 96 to base 207,
which is the exon/intron junction (in bold).

15 gtactggtgg agtatttgat agtgtattaa ccttatgtgt gacatgttct aatatagtca
cattttcatt atttttatta taaggcctgc tgaatatgac tgaatatataa ctgtggttag
ttggagctgg tggcgtaggc aagagtgctt tgacgataca gctaattcag aatcattttg
tggacgaata tgatccaaca atagaggtaa atcttgttt aatatgcata ttactggtgc
20 aggaccattc ttgatacag ataaaggttt ctctgacat ttcatgagt, SEQ ID NO:2.

The following sequence contains exon 2 from bases 358 to 536 (in bold).

-18-

atcaccattt tacattccca ccagcaatgc acaaagattt cagtgtctgt atccttgcta
 acacttattt tccatttttt gagttttttt gtttgtttt tttaataata gccaatccta
 atgggtatgt ggtagcatct catggttttg atttattttt cctgactatt gatgatgttg
 agcatctttt caggtgctta gtggccattt gtccgtcatc ttggagcag gaacaatgtc
 5 tttcaagtc cttgcccac ttttaattg aattttttgt tgttgagttg tatataacac
 cttttttgaa gtaaaagggtg cactgtaata atccagactg tgtttctccc ttctcaggat
 tcctacagga agcaagtagt aattgatgga gaaacctgtc tcttgatat tctcgacaca
 gcaggtcaag aggagtacag tgcaatgagg gaccagtaca tgaggactgg ggagggtttt
 ctttgtgtat ttgccataaa taatactaaa tcatttgaag atattcacoa ttatagggtg
 10 gtttaaatg aatataataa gctgacatta aggagtaatt atagttttta ttttttgagt
 ctttgctaag gccatgcata taatatatta taaaaatttt taaataatgt ttatgaggta
 ggtaatatcc ctgttttata aatgaagtgc ttgggggatt agagcagtgg agtaacttgc
 tccagactgc atcggtagtg gtggtgctgg gatgaaacc taggcctgtt tgactccaca
 gccttctgt, SEQ ID NO:3.

15

The following sequence contains exon 3, from base 299 to base 458 (in bold).

tctagaattt ttcagtagtt tctgtttttac tattatgac tacctgcata ttaacctatt
 aggttatagt ttactatac ttctaggtat ttgatctttt gagagagata caaggtttct
 20 gtttaaaaag gtaaaagaaac aaaataacta gtagaagaag gaaggaaaat ttggtgtagt
 ggaaactagg aattacattg ttttcttca gccaaatttt atgacaaaag ttgtggacag
 gttttgaaag atatttgtgt tactaatgac tgtgctataa ctttttttcc tttcccagag
 aacaaattaa **agaggttaag gactctgaag atgtacctat ggctcctagta ggaaataaat**

gtgatttgcc ttctagaaca gtagacacaa aacagggtcga ggacttagca agaagttatg
 gaatttocttt tattgaaaca tcagcaaga caagacagggt aagtaacact gaaataaata
 cagatctggt ttctgcaaaa tcataactgt tatgtcattt aatataatcag tttttctctc
 aattatgcta tactaggaaa taaaacaata ttagtaaat gttttgtct cttgagaggg
 5 cattgcttct taatc, SEQ ID NO:4.

The following sequence contains exon 4 from base 600 to base 723 (in bold).

acagaagacc cagtctcagc ttcacttgta taccctggaa atagactgaa aggtgttaaa
 10 atttaaaata aaactcaagg ttccagtttc ttgactcacc ttgagattc ttttatgttt
 ttgtgtttt ttaacaaaagg ttccacgtcc atattttacc attttcttc tcattctccc
 ctggaggagg gtgtgggaat cgatagtata taaatcactt ttttcctaag tcaagaaggt
 aatttaaagc taacttcagt ttaggcttta attccaggac tagcaaaacta aaatgggtgc
 attaattgac aaacagatgc taatacctgt gtttaggctt gtcataatct ctctaattc
 15 ctaatttaaa aatttttaaa tttaattcca ttgaaaaaca aaactgactt ttaagaacaa
 accaggattc tagcccatat tttaaaactg catcctcagt ttatttcaaa cagtctgatg
 tctgtttaaa aaaaaaaaaa tctcaagctc ataattctcaa acttcttgca catggctttc
 ccagtaaat actcttacca atgcaacaga ctttaaaaga gttgtgtttt acaatgcaga
 gagtggagga tgctttttat acatttggtga gagagatccg acaatacaga ttgaaaaaaa
 20 tcagcaaaaga agaaaagact cctgggtgtg tgaaaaattaa aaaatgcatt ataattgtaat
 ctggtaagtt taagtccagc acattaattt tggcagaaag cagatgtctt ttaaaggtaa
 caaggtggca accactttag aactacttag gtgtagtatt ctaacttgaa gtattaaaaag
 ataagaact tgttccata attagt, SEQ ID NO:5.

-20-

The following sequence contains exon 5 from base 159 to base 275 (in bold).

```

5  gaattctaaa agtccctaata tatgtaatat atattcagtt gcctgaagag aaacataaaag
   aatcctttct taatatatctt tccattaatg aaatttgta cctgtacaca tgaagccatc
   gtatatattc acattttaat actttttatg tatttcaggg tgttgatgat gccttctata
   cattagttcg agaatttcga aaacataaaag aaagatgag caaagatggg aaaaagaaga
   aaaagaagtc aaagacaaaag tgtgtaatta tgtaaataca atttgtactt ttttcttaag
   gcatactagt acaagtggta atttttgtac attacactaa attattagca ttgtttttag
   cattacctaa ttttttctt gctccatgca gactgttagc ttttaccttaaatgcttatt
   ttaaaatgac agtggaagtt ttttttctt cgaagtgccg gtattcccag agttttgggtt
   tttgaactag caatgcctgt gaaaaagaaa ctgaatacct aagattttctg tcttgggggtt
   tttggtgcat gcagttgatt acttcttatt tttcttacca agtgtgaatg ttggtgtgaa
   acaaaattaat gaagctt, SEQ ID NO:6.
10

```

15 The following sequence is a cDNA sequence of K-ras and the derived amino acid sequence.

```

20 atg act gaa tat aaa ctt gtg gta gtt gga gct ggt ggc gta ggc aag agt
   M T E Y K L V V V G A G G V G K S
   gcc ttg acg ata cag cta att cag aat cat ttt gtg gac gaa tat gat cca
   A L T I Q L I Q N H F V D E Y D P
   aca ata gag gat tcc tac agg aag caa gta gta att gat gga gaa acc tgt
   T I E D S Y R K Q V V I D G E T C

```

```

ctc ttg gat att ctc gac aca gca ggt caa gag gag tac agt gca atg agg
L L D I L D T A G Q E E Y S A M R
gac cag tac atg agg act ggg gag ggc ttt ctt tgt gta ttt gcc ata aat
D Q Y M R T G E G F L C V F A I N
5 aat act aaa tca ttt gaa gat att cac cat tat aga gaa caa att aaa aga
N T K S F E D I H H Y R E Q I K R
gtt aag gac tct gaa gat gta cct atg gtc cta gta gga aat aaa tgt gat
V K D S E D V P M V L V G N K C D
ttg cct tct aga aca gta gac aca aaa cag gct cag gac tta gca aga agt
10 L P S R T V D T K Q A Q D L A R S
tat gga att cct ttt att gaa aca tca gca aag aca aga cag aga gtg gag
Y G I P F I E T S A K T R Q R V E
gat gct ttt tat aca ttg gtg aga gag atc cga caa tac aga ttg aaa aaa
D A F Y T L V R E I R Q Y R L K K
15 atc agc aaa gaa aag act cct ggc tgt gtg aaa att aaa aaa tgc att
I S K E E K T P G C V K I K K C I
ata atg taa tct g, SEQ ID NO:7.
I M *, SEQ ID NO:8.

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B. ADENOVIRAL EXPRESSION VECTORS

Throughout this application, the term "adenoviral expression vector" is meant to include those constructs
5 containing adenovirus sequences sufficient to (i) support packaging of the construct and (ii) to express an antisense polynucleotide that has been cloned therein. In this context, expression does not require that the gene product be synthesized.

10

The expression vector comprises a genetically engineered form of adenovirus. Knowledge of the genetic organization of adenovirus, a 36 kB, linear and double-stranded DNA virus, allows substitution of a large
15 piece of adenoviral DNA with foreign sequences up to 7 kB (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the infection of adenoviral DNA into host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential
20 genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be
25 linked only to mild disease such as acute respiratory disease in the human.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized genome,
30 ease of manipulation, high titer, wide target-cell range, and high infectivity. Both ends of the viral genome contain 100-200 base pair (bp) inverted terminal repeats (ITR), which are cis elements necessary for viral DNA replication and packaging. The early (E) and late (L)
35 regions of the genome contain different transcription units that are divided by the onset of viral DNA

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replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression, and host cell shut off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNA's issued from this promoter possess a 5' tripartite leader (TL) sequence which makes them preferred mRNA's for translation.

In the current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure. Use of the YAC system is an alternative approach for the production of recombinant adenovirus.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Ad5 DNA fragments and constitutively expresses E1 proteins (Graham, et al., 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the E3 or

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both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury, et al., 1987), providing capacity for about 2 extra kB of DNA. Combined
5 with the approximately 5.5 kB of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kB, or about 15% of the total length of the vector. More than 80% of the
10 adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1 deleted virus is incomplete. For example, leakage of viral gene
expression has been observed with the currently available adenovirus vectors at high multiplicities of infection
15 (Mulligan, 1993).

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal
20 or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, e.g., Vero cells or other monkey embryonic mesenchymal or
epithelial cells. As stated above, the preferred helper
25 cell line is 293.

Recently, Racher et al. (1995) disclosed improved methods for culturing 293 cells and propagation of adenovirus. In one format, natural cell aggregates are
30 grown by inoculating individual cells into 1 L siliconized spinner-flasks (Techne, Cambridge, UK) containing 100-200 ml of medium. Following stirring at 40 rpm, the cell viability is estimated with trypan blue. In another format, Fibra-Cel microcarriers (Bibby
35 Sterlin, Stone, UK) (5 g/l) is employed as follows. A cell inoculum, resuspended in 5 ml of medium, is added to

- 25 -

the carrier (50 ml) in a 250 ml Erlenmeyer flask and left stationary, with occasional agitation, for 1-4 h. The medium is then replaced with 50 ml of fresh medium and shaking started. For virus production, cells are allowed to grow to about 80% confluence after which time the medium is replaced (to 25% of the final volume) and adenovirus added at an MOI of 0.05. Cultures are left stationary overnight, following which the volume is increased to 100% and shaking commenced for another 72 h.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the method of the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the polynucleotide encoding K-ras at the position from which the E1 coding sequences have been removed. However, the position of insertion of the K-ras construct within the adenovirus sequences is not critical to the present invention. The polynucleotide encoding a K-ras antisense transcription unit also may be inserted in lieu of the deleted E3 region in E3 replacement vectors as described previously by Karlsson

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et. al. (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus is easy to grow and manipulate and
5 exhibits broad host range *in vitro* and *in vivo*. This group of viruses can be obtained in high titers, e.g., 10^9 - 10^{11} plaque-forming unit (PFU)/ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The
10 foreign genes delivered by adenovirus vectors are episomal, and therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination with wild-type adenovirus (Couch et al., 1963; Top et al., 1971), demonstrating their safety and
15 therapeutic potential as *in vivo* gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero et al., 1991; Gomez-Foix et al., 1992) and vaccine development (Grunhaus and Horwitz,
20 1992; Graham and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet et al., 1990; Rich et al., 1993). Studies in administering recombinant adenovirus
25 to different tissues include trachea instillation (Rosenfeld et al., 1991; Rosenfeld et al., 1992), muscle injection (Ragot et al., 1993), peripheral intravenous injection (Herz and Gerard, 1993), and stereotactic inoculation into the brain (Le Gal La Salle et al.,
30 1993).

The polynucleotide encoding the K-ras polynucleotide typically is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the
35 synthetic machinery of the host cell, or introduced synthetic machinery, that is required to initiate the

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specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location in relation to the polynucleotide to control RNA polymerase initiation and expression of the polynucleotide.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved

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relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can
5 function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a K-ras polynucleotide is not believed
10 to be critical, so long as it is capable of expressing the polynucleotide in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the polynucleotide coding region adjacent to and under the control of a promoter that is capable of being expressed
15 in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early
20 promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of the K-ras polynucleotide. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to achieve expression of
25 polynucleotides is contemplated as well, provided that the levels of expression are sufficient to produce a growth inhibitory effect.

By employing a promoter with well-known properties,
30 the level and pattern of expression of a polynucleotide following transfection can be optimized. For example, selection of a promoter which is active in specific cells, such as tyrosinase (melanoma), alpha-fetoprotein and albumin (liver tumors), CC10 (lung tumor) and
35 prostate-specific antigen (prostate tumor) will permit tissue-specific expression of K-ras antisense

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polynucleotides. Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of the antisense construct. For example, with the polynucleotide under the control of the human PAI-1 promoter, expression is inducible by tumor necrosis factor. Tables 2 and 3 list several elements/promoters which may be employed, in the context of the present invention, to regulate the expression of K-ras antisense constructs. This list is not intended to be exhaustive of all the possible elements involved in the promotion of K-ras antisense expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

- 30 -

Below is a list of viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in the K-ras antisense polynucleotide expression vector (Table 2 and Table 3). Additionally

5 any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of a K-ras construct. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic

10 transcription from certain bacteriophage promoters if the appropriate bacteriophage polymerase is provided, either as part of the delivery complex or as an additional genetic expression vector.

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TABLE 2

ENHANCER	
5	Immunoglobulin Heavy Chain
	Immunoglobulin Light Chain
	T-Cell Receptor
	HLA DQ α and DQ β
	β -Interferon
10	Interleukin-2
	Interleukin-2 Receptor
	MHC Class II 5 α
	MHC Class II HLA-DR α
	β -Actin
15	Muscle Creatine Kinase
	Prealbumin (Transthyretin)
	Elastase I
	Metallothionein
	Collagenase
20	Albumin Gene
	α -Fetoprotein
	γ -Globin
	β -Globin
	c-fos
25	c-HA-ras
	Insulin
	Neural Cell Adhesion Molecule (NCAM)
	α 1-Antitrypsin
	H2B (TH2B) Histone
30	Mouse or Type I Collagen
	Glucose-Regulated Proteins (GRP94 and GRP78)
	Rat Growth Hormone
	Human Serum Amyloid A (SAA)

TABLE 2 (continued)

ENHANCER	
	Troponin I (TN I)
	Platelet-Derived Growth Factor
	Duchenne Muscular Dystrophy
	SV40
5	Polyoma
	Retroviruses
	Papilloma Virus
	Hepatitis B Virus
	Human Immunodeficiency Virus
10	Cytomegalovirus
	Gibbon Ape Leukemia Virus

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TABLE 3

	Element	Inducer
5	MT II	Phorbol Ester (TFA) Heavy metals
	MMTV (mouse mammary tumor virus)	Glucocorticoids
	β -Interferon	poly(rI)X poly(rc)
	Adenovirus 5 <u>E2</u>	Ela
10	c-jun	Phorbol Ester (TPA), H ₂ O ₂
	Collagenase	Phorbol Ester (TPA)
	Stromelysin	Phorbol Ester (TPA), IL-1
	SV40	Phorbol Ester (TPA)
	Murine MX Gene	Interferon, Newcastle Disease Virus
15	GRP78 Gene	A23187
	α -2-Macroglobulin	IL-6
	Vimentin	Serum
	MHC Class I Gene H-2kB	Interferon
20	HSP70	Ela, SV40 Large T Antigen
	Proliferin	Phorbol Ester-TPA
	Tumor Necrosis Factor	FMA
25	Thyroid Stimulating Hormone α Gene	Thyroid Hormone

In certain embodiments of the invention, the delivery of an expression vector in a cell may be identified *in vitro* or *in vivo* by including a marker in the expression vector. The marker would result in an identifiable change to the transfected cell permitting easy identification of expression. Usually the inclusion

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of a drug selection marker aids in cloning and in the selection of transformants. Alternatively, enzymes such as herpes simplex virus thymidine kinase (*tk*) (eukaryotic) or chloramphenicol acetyltransferase (CAT) (prokaryotic) may be employed. Immunologic markers also can be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed along with the polynucleotide encoding K-ras antisense. Further examples of selectable markers are well known to one of skill in the art.

One will typically include a polyadenylation signal to effect proper polyadenylation of the antisense transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. The inventors have employed the SV40 polyadenylation signal in that it was convenient and known to function well in the target cells employed. Also contemplated as an element of the expression construct is a terminator. These elements can serve to enhance message levels and to minimize read through from the construct into other sequences.

25 C. METHODS FOR GENE TRANSFER

In order to effect expression of antisense K-ras constructs, the expression vector must be delivered into a cell. As described above, the preferred mechanism for delivery is via viral infection where the expression vector is encapsidated in an infectious adenovirus particle.

Several non-viral methods for the transfer of expression vectors into cultured mammalian cells also are contemplated by the present invention. These include

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calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe et al., 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa et al., 1986; Potter et al., 1984), direct microinjection
5 (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley et al., 1979) and lipofectamine-DNA complexes, cell sonication (Fechheimer et al., 1987), gene bombardment using high velocity microprojectiles (Yang et al., 1990), polycations
10 (Boussif et al., 1995) and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

15 In one embodiment of the invention, the adenoviral expression vector may simply consist of naked recombinant vector. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. For example,
20 Dubensky et al. (1984) successfully injected polyomavirus DNA in the form of CaPO_4 precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct
25 intraperitoneal injection of CaPO_4 precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding an antisense K-ras construct may also be transferred in a similar manner *in vivo*.

30 Another embodiment of the invention for transferring a naked DNA expression vector into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter
35 cells without killing them (Klein et al., 1987). Several devices for accelerating small particles have been

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developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang et al., 1990). The microprojectiles used have consisted of biologically
5 inert substances such as tungsten or gold beads.

Selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded in vivo (Yang et al., 1990; Zelenin et al., 1991). This may
10 require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun and the target organ. DNA encoding a K-ras antisense construct may be delivered via this method.

15 In a further embodiment of the invention, the expression vector may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid
20 layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between
25 the lipid bilayers (Ghosh and Bachhawat, 1991). Also contemplated are lipofectamine-DNA complexes.

Liposome-mediated polynucleotide delivery and expression of foreign DNA in vitro has been very
30 successful. Wong et al. (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa and hepatoma cells. Nicolau et al. (1987) accomplished successful liposome-mediated gene transfer in rats after
35 intravenous injection.

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In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of

5 liposome-encapsulated DNA (Kaneda et al., 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato et al., 1991). In yet

10 further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression vectors have been successfully employed in transfer and expression of a polynucleotide in vitro and in vivo, then they are applicable for the present invention. Where a bacteriophage promoter is employed in

15 the DNA construct, it also will be desirable to include within the liposome an appropriate bacteriophage polymerase.

Another mechanism for transferring expression

20 vectors into cells is receptor-mediated delivery. This approach takes advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific distribution of various receptors, the delivery can be

25 highly specific (Wu and Wu, 1993). Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively

30 characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner et al., 1993). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol et al., 1993; Perales et al.,

35 1994) and epidermal growth factor (EGF) has also been

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used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may
5 comprise a ligand and a liposome. For example, Nicolau
et al. (1987) employed lactosyl-ceramide, a
galactose-terminal asialoganglioside, incorporated into
liposomes and observed an increase in the uptake of the
insulin gene by hepatocytes. Thus, it is feasible that
10 an adenoviral expression vector also may be specifically
delivered into a cell type such as lung, epithelial or
tumor cells, by any number of receptor-ligand systems,
with or without liposomes. For example, epidermal growth
factor (EGF) may be used as the receptor for mediated
15 delivery of K-ras construct in many tumor cells that
exhibit upregulation of EGF receptor. Mannose can be
used to target the mannose receptor on liver cells.
Also, antibodies to CD5 (CLL), CD22 (lymphoma), CD25
(T-cell leukemia) and MAA (melanoma) can similarly be
20 used as targeting moieties.

In certain embodiments, gene transfer may more
easily be performed under *ex vivo* conditions. *Ex vivo*
gene therapy refers to the isolation of cells from an
25 animal, the delivery of a polynucleotide into the cells,
in vitro, and then the return of the modified cells back
into an animal. This may involve the surgical removal of
tissue/organs from an animal or the primary culture of
cells and tissues. Anderson et al., U.S. Patent
30 5,399,346, and incorporated herein in its entirety,
disclose *ex vivo* therapeutic methods. During *ex vivo*
culture, the expression vector can express the antisense
K-ras construct. Finally, the cells may be reintroduced
into the original animal, or administered into a distinct
35 animal, in a pharmaceutically acceptable form by any of
the means described below.

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**D. K-ras EXPRESSION VECTORS IN COMBINATION WITH
OTHER THERAPIES**

Tumor cell resistance to DNA damaging agents
5 represents a major problem in clinical oncology. One
goal of current cancer research is to find ways to
improve the efficacy of chemo- and radiotherapy by
combining it with gene therapy. For example, the herpes
simplex-thymidine kinase (HS-tK) gene, when delivered to
10 brain tumors by a retroviral vector system, successfully
induced susceptibility to the antiviral agent ganciclovir
(Culver, et al., 1992). In the context of the present
invention, it is contemplated that antisense K-ras
therapy could be used similarly in conjunction with
15 chemo- or radiotherapeutic intervention.

To kill cells, such as malignant or metastatic
cells, using the methods and compositions of the present
invention, one would generally contact a "target" cell
20 with an expression vector and at least one DNA damaging
agent. These compositions would be provided in a
combined amount effective to kill or inhibit
proliferation of the cell. This process may involve
contacting the cells with the expression vector and the
25 DNA damaging agent(s) or factor(s) at the same time.
This may be achieved by contacting the cell with a single
composition or pharmacological formulation that includes
both agents, or by contacting the cell with two distinct
compositions or formulations, at the same time, wherein
30 one composition includes the K-ras expression vector and
the other includes the DNA damaging agent.

Alternatively, the K-ras treatment may precede or
follow the DNA damaging agent treatment by intervals
35 ranging from minutes to weeks. In embodiments where the
DNA damaging factor and K-ras expression vector are

- 40 -

applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the DNA damaging agent and expression vector would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with both agents within about 12-24 h of each other and, more preferably, within about 6-12 h of each other, with a delay time of only about 12 h being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

15

It also is conceivable that more than one administration of either the K-ras construct or the DNA damaging agent will be desired. Various combinations may be employed, where K-ras is "A" and the DNA damaging agent is "B":

20

A/B/A	B/A/B	B/B/A	A/A/B	B/B/B/A	B/B/A/B
A/A/B/B	A/B/A/B	A/B/B/A	B/B/A/A	B/A/B/A	B/A/A/B
A/A/A/B	B/A/A/A	A/B/A/A	A/A/B/A	A/B/B/B	B/A/B/B

25

To achieve cell killing, both agents are delivered to a cell in a combined amount effective to kill the cell.

DNA damaging agents or factors are defined herein as any chemical compound or treatment method that induces DNA damage when applied to a cell. Such agents and factors include radiation and waves that induce DNA damage such as, γ -irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, and the like. A variety of chemical compounds, also described as

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"chemotherapeutic agents", function to induce DNA damage, all of which are intended to be of use in the combined treatment methods disclosed herein. Chemotherapeutic agents contemplated to be of use, include, e.g.,
5 adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP) and even hydrogen peroxide. The invention also encompasses the use of a combination of one or more DNA
10 damaging agents, whether radiation-based or actual compounds, such as the use of X-rays with cisplatin or the use of cisplatin with etoposide. In certain embodiments, the use of cisplatin in combination with a K-ras antisense expression vector is particularly preferred.

15

In treating cancer according to the invention, one would contact the tumor cells with a DNA damaging agent in addition to the expression vector. This may be achieved by irradiating the localized tumor site with DNA
20 damaging radiation such as X-rays, UV-light, γ -rays or even microwaves. Alternatively, the tumor cells may be contacted with the DNA damaging agent by administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a DNA damaging
25 compound such as, adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, or more preferably, cisplatin. The DNA damaging agent may be prepared and used as a combined therapeutic composition, or kit, by combining it with a K-ras expression vector,
30 as described above.

Agents that directly cross-link polynucleotides, specifically DNA, are envisaged and are shown herein, to eventuate DNA damage leading to a synergistic
35 antineoplastic combination. Agents such as cisplatin, and other DNA alkylating may be used. Cisplatin has been

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widely used to treat cancer, with efficacious doses used in clinical applications of 20 mg/m² for 5 days every three weeks for a total of three courses. Cisplatin is not absorbed orally and must therefore be delivered via injection intravenously, subcutaneously, intratumorally or intraperitoneally.

Agents that damage DNA also include compounds that interfere with DNA replication, mitosis and chromosomal segregation. Such chemotherapeutic compounds include adriamycin, also known as doxorubicin, etoposide, verapamil, podophyllotoxin, and the like. Widely used in a clinical setting for the treatment of neoplasms, these compounds are administered through bolus injections intravenously at doses ranging from 25-75 mg/m² at 21 day intervals for adriamycin, to 35-50 mg/m² for etoposide intravenously or double the intravenous dose orally.

Agents that disrupt the synthesis and fidelity of polynucleotide precursors and subunits also lead to DNA damage. As such a number of polynucleotide precursors have been developed. Particularly useful are agents that have undergone extensive testing and are readily available. As such, agents such as 5-fluorouracil (5-FU), are preferentially used by neoplastic tissue, making this agent particularly useful for targeting to neoplastic cells. Although quite toxic, 5-FU, is applicable in a wide range of carriers, including topical, however intravenous administration with doses ranging from 3 to 15 mg/kg/day being commonly used.

Other factors that cause DNA damage and have been used extensively include what are commonly known as γ -rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves

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and UV-irradiation. It is most likely that all of these factors effect a broad range of DNA damage, or the precursors of DNA, the replication and repair of DNA, and the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 weeks), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

The skilled artisan is directed to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 33, in particular pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

The inventors propose that the regional delivery of K-ras expression vectors to patients with K-ras-linked cancers will be a very efficient method for delivering a therapeutically effective gene to counteract the clinical disease. Similarly, the chemo- or radiotherapy may be directed to a particular, affected region of the subject's body. Alternatively, systemic delivery of the expression vector or the DNA damaging agent may be appropriate in certain circumstances, for example, where extensive metastasis has occurred.

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Cytokine therapy also has proven to be an effective partner for combined therapeutic regimens. Various cytokines may be employed in such combined approaches. Examples of cytokines include IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, TGF- β , GM-CSF, M-CSF, G-CSF, TNF α , TNF β , LAF, TCGF, BCGF, TRF, BAF, BDG, MP, LIF, OSM, TMF, PDGF, IFN- α , IFN- β , IFN- γ . Cytokines are administered according to standard regimens, as described below, consistent with clinical indications such as the condition of the patient and relative toxicity of the cytokine.

In addition to combining anti-K-ras-targeted therapies with chemo-, radio- and cytokine therapies, it also is contemplated that combination with other gene therapies will be advantageous. For example, targeting of K-ras and p53 mutations at the same time may produce an improved anti-cancer treatment. Any other tumor-related gene conceivably can be targeted in this manner, for example, p53, p21, Rb, APC, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, FCC, MCC, other *ras* molecules, *myc*, *neu*, *raf*, *erb*, *src*, *fms*, *jun*, *trk*, *ret*, *gsp*, *hst*, *bcl* and *abl*. It also may be desirable to combine anti-sense K-ras therapy with an antibody-based gene therapy treatment involving the use of a single-chain antibody construct in which the antibody binds to any of the foregoing molecules.

E. PHARMACEUTICAL COMPOSITIONS AND ROUTES OF ADMINISTRATION

Where clinical application of an adenoviral expression according to the present invention is contemplated, it will be necessary to prepare the complex as a pharmaceutical composition appropriate for the

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intended application. Generally this will entail preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals.

- 5 One also will generally desire to employ appropriate salts and buffers to render the complex stable and allow for complex uptake by target cells.

Aqueous compositions of the present invention
10 comprise an effective amount of the expression vector, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrases "pharmaceutically or pharmacologically acceptable" refer to molecular entities
15 and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial
20 and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in
25 the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

Solutions of the active compounds as free base or
30 pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, mixtures thereof and in oils. Under ordinary conditions of
35 storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

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The expression vectors and delivery vehicles of the present invention may include classic pharmaceutical preparations. Administration of therapeutic compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration will be by orthotopic, intradermal subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients.

The therapeutic compositions of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain 10 mg, 25 mg, 50 mg or up to about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the

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various components the pharmaceutical composition are adjusted according to well known parameters.

Additional formulations are suitable for oral administration. Oral formulations include such typical excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. When the route is topical, the form may be a cream, ointment, salve or spray.

An effective amount of the therapeutic agent is determined based on the intended goal, for example (i) inhibition of tumor cell proliferation or (ii) elimination of tumor cells. The term "unit dose" refers to physically discrete units suitable for use in a subject, each unit containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses, discussed above, in association with its administration, i.e., the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the subject to be treated, the state of the subject and the protection desired. Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual.

In certain embodiments, it may be desirable to provide a continuous supply of therapeutic compositions to the patient. For intravenous or intraarterial routes, this is accomplished by drip system. For topical applications, repeated application would be employed. For various approaches, delayed release formulations could be used that provided limited but constant amounts

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of the therapeutic agent over and extended period of time. For internal application, continuous perfusion of the region of interest may be preferred. This could be accomplished by catheterization, post-operatively in some cases, followed by continuous administration of the therapeutic agent. The time period for perfusion would be selected by the clinician for the particular patient and situation, but times could range from about 1-2 h, to 2-6 h, to about 6-10 h, to about 10-24 h, to about 1-2 days, to about 1-2 weeks or longer.

F. KITS

All the essential materials and reagents required for inhibiting tumor cell proliferation may be assembled together in a kit. This generally will comprise selected adenoviral expression vectors. Also included may be various media for replication of the expression vectors and host cells for such replication. Such kits will comprise distinct containers for each individual reagent.

When the components of the kit are provided in one or more liquid solutions, the liquid solution preferably is an aqueous solution, with a sterile aqueous solution being particularly preferred. For *in vivo* use, the expression vector may be formulated into a pharmaceutically acceptable syringeable composition. In this case, the container means may itself be an inhalent, syringe, pipette, eye dropper, or other such like apparatus, from which the formulation may be applied to an infected area of the body, such as the lungs, injected into an animal, or even applied to and mixed with the other components of the kit.

The components of the kit may also be provided in dried or lyophilized forms. When reagents or components

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are provided as a dried form, reconstitution generally is by the addition of a suitable solvent. It is envisioned that the solvent also may be provided in another container means.

5

The kits of the present invention also will typically include a means for containing the vials in close confinement for commercial sale such as, e.g., injection or blow-molded plastic containers into which
10 the desired vials are retained. Irrespective of the number or type of containers, the kits of the invention also may comprise, or be packaged with, an instrument for assisting with the injection/administration or placement of the ultimate complex composition within the body of an
15 animal. Such an instrument may be an inhalent, syringe, pipette, forceps, measured spoon, eye dropper or any such medically approved delivery vehicle.

G. EXAMPLES

20

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow
25 represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many
30 changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

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Example 1 - Materials and Methods

Cell Lines and Culture Conditions. 293 cells (293S, human embryonic kidney cells) at passage thirty-one, grown in minimal essential medium with nonessential amino acids and 10% horse serum, were used for cotransfections. A selected population of 293 cells with faster growing properties (293F) was grown in DMEM⁴ with 10% FBS and used for virus amplification. The human NSCLC cell line H460a was maintained in RPMI medium with 5% fetal bovine serum (FBS). This cell line was derived from a large-cell undifferentiated NSCLC line and contains a homozygous mutation at codon 61 of K-ras (Mukhopadhyay et al., 1991). To infect 293 and H460a cells with recombinant adenovirus, subconfluent cell monolayers were first incubated with the virus in a minimal amount of complete medium (1 ml/60-mm plate, 37°C in CO₂ incubator, 1 h rocking plates every 10 min to avoid drying). Complete medium was then added and the plates were incubated for 16 h or, in the case of 293 cells, until the appearance of cytopathic effect.

Generation of Recombinant Adenoviruses. An Nde I-Sal I genomic fragment from the K-ras protooncogene, containing exons 2 (176 bp) and 3 (130 bp) with flanking intron sequences and complete intron 2 (1.7 kB) was obtained from the plasmid Apr1-neo-Kras (Mukhopadhyay et al., 1991). After blunting the ends with the Klenow, the fragment was cloned between the CMV promoter and SV40 poly A signal in both sense (S) and antisense (AS) orientations. These two expression cassettes and an empty one (to generate another adenoviral vector used as a control) were excised by complete Cla I digestion and partial Xba I digestion (there is an Xba I site in the K-ras fragment) and inserted into the polylinker of plasmid pXCJL.1 (F. Graham, McMaster University,

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Hamilton, Ontario, Canada) using Xba I and Cla I. The resulting adenoviral shuttle vectors were cotransfected with pJM17 in 293 cells by DOTAP lipofection (Zhang et al., 1993) to generate AdKrasS (sense), AdKrasAS (antisense) and AdCMV-pA (empty) vectors. Viruses were subsequently plaque-isolated on 293S cells and amplified in 293F by standard procedures (Zhang et al., 1994; Graham and Prevec, 1991). The viruses were purified by two CsCl gradients (a step gradient of 1.5-1.35-1.25 g/ml, 150,000g 1 h and a continuous gradient of 1.35 g/ml, 150,000g 16 h). After dialysis, stocks were kept at -80°C in a solution containing 10 mM Tris-HCl, pH 7.5; 1 mM MgCl₂; and 10% glycerol. Titers of purified viruses were determined by plaque assays (Graham and Prevec, 1991).

Northern Blot. Total cellular RNA was isolated from cells with Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH). Twenty micrograms of RNA were size-fractionated in MOPS/formaldehyde gels and vacuum transferred to Zeta-Probe GT blotting membranes (BioRad, Hercules, CA). Hybridization and washing were performed according to the manufacturer's instructions. The 2 kB genomic fragment from K-ras, labeled by random primer (Redi-prime; Amersham, Piscataway, NJ) at 2x10⁶ cpm/ml, was used as a probe.

Western Blot. Lysis of infected cells, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of fractions, size fractionation, transfer, and immunodetection were performed as described elsewhere (Zhang et al., 1994). The antibodies used were: F234-4.2 anti-c-K-ras monoclonal antibody (Oncogene Science, Nanhasset, NY) diluted at 1/15; anti-actin monoclonal antibody (Amersham) diluted at 1/3000; and an anti-mouse immunoglobulin horseradish peroxidase-linked

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whole antibody from sheep (Amersham, San Diego, CA)
diluted at 1/3000.

Proliferation and Colony Formation Assays. For
5 [3H]thymidine uptake assays, cells grown at 50-60%
confluence in 60-mm plates were infected for 24 h,
trypsinized, counted and seeded in triplicate 96-well
plates at 1×10^3 cells/well. At the specified day, 10 μ l
of a 1:10 dilution of [3H]thymidine (5 Ci/mmol, Amersham)
10 in DMEM with 3% FBS was added to each well and incubated
for 4 h. Then cells were washed and harvested to filters
for radioactivity counting. Direct cell number assays
were performed as described elsewhere (Zhang et al.,
1994).

15

For soft agarose colony formation assays, infected
cells were trypsinized, mixed with 0.35% agarose and
plated over a base layer of 0.7% agarose as described
elsewhere (Zhang et al., 1993). Colonies were counted 10
20 days later.

Example 2 - Generation of AdKras and AdKrasAS

To construct a recombinant adenovirus expressing a
25 mutated K-ras, a 2 kB fragment was inserted downstream of
a strong promoter. This fragment was chosen because it
has been shown to block p21 protein expression in other
systems without affecting the expression of the other
proteins of the ras family (Zhang et al., 1993). The
30 steps used to construct the virus are parallel to those
used to generate the adenoviral vector Ad5CMV-p53 (Zhang
et al., 1994). First, the fragment is inserted in an
expression cassette. Second, this cassette is inserted
into the E1-deleted region of the Ad5 left arm. And
35 third, this construct is cotransfected with a
nonpackageable Ad genome (pJM17). FIG. 1 shows these

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steps schematically. The structure of the virus so produced was confirmed by restriction analysis. The Xba I sites at the end of exon 3 in the K-ras fragment and in front of the CMV promoter allows clear distinction
5 between the sense and the antisense constructs.

Example 3 - Expression of Antisense K-ras RNA in Infected Cells

10 The first step in assessing the effect of AdKrasAS is to define an appropriate range of dose and toxicity. It was assumed that the more antisense RNA present in the cell, the stronger the growth inhibitory-effects would be, with a limit imposed by the toxic effects of large
15 doses of viral proteins. To determine the optimal therapeutic ratio, H460a cells were infected with an adenovirus expressing the β -gal gene (Ad5CMV-LacZ; Zhang et al., 1994), at an increasing multiplicity of infection (MOI). At an MOI of 100 pfu/cell, 65% of cells were
20 transduced with the β -gal gene, as determined by X-gal staining. To reach a complete transduction of H460a cells, an MOI of about 1000 pfu/cell was required. In preliminary tests of toxicity in H460a cells using several control viruses such as Ad5CMV-LacZ, AdKrasS and
25 AdCMV-pA, it was found that MOI's higher than 400 pfu/cell reduced cell proliferation. Taking these observations into account, an MOI of 100 pfu/cell was chosen to test the effect of AdKrasAS.

30 To determine the effects of AdKrasAS at the mRNA level, cells were infected with AdKrasS or with AdKrasAS, and total RNA extracted after 1, 3 and 5 days was analyzed by Northern blot. Cells infected with the control virus AdKrasS expressed the same K-ras RNA levels
35 as noninfected cells. The size of the transcript that arose from AdKrasS appeared to be approximately 4 kB,

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which indicates a possible read-through of the SV40 polyA signal. In cells infected with the antisense AdKrasAS virus, the endogenous K-ras RNA transcript detected in noninfected cells was no longer detected, and other
5 transcripts of approximately 4 and 6 kB appeared, presumable arising from AdKrasAS. These results, reproducibly confirmed, indicate that AdKrasAS affects the pattern of K-ras mRNA expression.

10 Protein production was analyzed by Western blot using a monoclonal antibody specific for the p21 protein. Three days after infection with AdKrasAS at an MOI of 100 pfu/cell (65% of cells transduced), the level of p21
15 protein was less than half (30%) of that found in uninfected cells or in cells infected with AdKrasS or AdCMV-LacZ. As an internal control, an anti-actin-specific antibody was added in the incubation step. Actin levels were the same irrespective of the treatment, indicating that the observed reduction in p21
20 protein was specific to the antisense virus.

Example 4 - Growth Inhibition by AdKrasAS

If translation of the p21 protein was blocked by the
25 antisense RNA from AdKrasAS, it would be expected that the virus would have a growth-inhibitory effect. To examine this, the growth of a population of cells infected with AdKrasAS was compared to that of uninfected cells, or infected with control virus. In wells to which
30 AdKrasAS was added, cell number and [³H]thymidine incorporation in a 4 h period were consistently (three independent studies) about 50% of those in controls (FIG. 2). After 4 days, although the cell number was lower, the proliferation rate of the remaining cells was the
35 same as the uninfected or control-infected populations, suggesting that these cells arose from non-transduced

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cells. No cells were observed for more than 5 days, because in control treatments, the monolayer was reaching confluence and cell number and thymidine incorporation began to plateau after this period. When an MOI higher than 400 pfu/cell was used in an attempt to transduce all cells, cell number and thymidine incorporation proportionally decreased in wells with control viruses. The observed magnitudes of nonspecific toxicity were: Ad5CMV-LacZ > AdCMV-pA > AdKrasS.

10

Another approach used to study the growth-inhibitory effect of AdKrasAS was to test the colony-forming ability of transduced cells. Plates with H460a cells infected with AdKrasAS consistently (three studies) showed about ten-fold fewer colonies; most cells remained as single cells (number of colonies, 121 ± 24), as compared with uninfected cells (1304 ± 182), AdKrasS-infected cells (1275 ± 165) and Ad5CMV-LacZ-infected cells (118 ± 134). Thus, AdKrasAS markedly decreased the capacity of human lung cancer cells to achieve anchorage-independent growth.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

5 (i) APPLICANT:

(A) NAME: BOARD OF REGENTS, THE UNIVERSITY OF
TEXAS SYSTEM

(B) STREET: 201 WEST 7TH STREET

(C) CITY: AUSTIN

10 (D) STATE: TEXAS

(E) COUNTRY: USA

(F) POSTAL CODE (ZIP): 78701

15 (ii) TITLE OF INVENTION: ADENOVIRUS-ANTISENSE K-RAS
EXPRESSION VECTORS AND
THEIR APPLICATION IN CANCER
THERAPY

(iii) NUMBER OF SEQUENCES: 8

20

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

25 (D) SOFTWARE: PatentIn Release #1.0, Version
#1.30 (EPO)

(vi) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 08/550,959

30 (B) FILING DATE: 31-OCT-1995

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 3220 base pairs

(B) TYPE: nucleic acid

- 68 -

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5
15
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ATTCTCGACA CAGCAGGTCA AGAGGAGTAC AGTGCAATGA GGGACCAGTA CATGAGGACT 720
GGGGAGGGCT TTCTTTGTGT ATTTGCCATA AATAATACTA AATCATTTGA AGATATTAC 780
CATTATAGGT GGGTTTAAAT TGAATATAAT AAGCTGACAT TAAGGAGTAA TTATAGTTTT 840
TATTTTTTGA GTCTTTGCTA ATGCCATGCA TATAATATTT AATAAAAATT TTAAAAAAT 900
GTTTATGAGG TAGGTAATAT CCTGTGTTTTA TAAATGAAAGT TCTTGGGGGA TTAGAGCAGT 960
GGAGTAACTT GCTCCAGACT GCATCGGTAG TGGTGGTGCT GGGATTGAAA CCTAGGCCCTG 1020
TTTGACTCCA CAGCCTTCTG TACTCTTGAC TATTCTACAA AAGCAAGACT TTAAACTTTT 1080
TAGATACATC ATTAAAAAAG AAAACCATAA AAAAGAATAT GAAAAGATGA TTGAGATGGT 1140
GTCACTTAAC AGTCTTAAAC AATCGGTGTA TAGCATAGAA TGCTGGATTG GATAAACCCAG 1200
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-69-

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15
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1320
1380
1440
1500
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1620
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1920
1980

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TGTAATTICA CCCCAAAATT AGAGTTTATG CACTTGTAT ATTGAAAATG ATTAACATGT
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-70-

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5 CCGGGCCGGC GCGTTATTAC CATAAAGGC AAACACTGGT CGGAGGCGTC CCGCGGGCGC 2160
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-71-

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20 (2) INFORMATION FOR SEQ ID NO: 2:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 290 base pairs

-72-

- (B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:
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TTGAGCTGG TGGCGTAGGC AAGAGTGCCT TGACGATACA GCTAATTCAG AATCATTTTG 180
TGGACGAATA TGATCCAACA ATAGAGGTAA ATCTTGTTTT AATATGCATA TTACTGGTGC 240
15 AGGACCATTCT TTGATACAG ATAAGGTTT CTCTGACCAT TTTCATGAGT 290

(2) INFORMATION FOR SEQ ID NO: 3:

- 20 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 789 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

-73-

(D) TOPOLOGY: linear

(x1) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

5	ATCACCAATT TACATTCCCA CCAGCAATGC ACAAGATTT CAGTGTCTGT ATCCTTGCTA	60
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	ATGGGTATGT GGTAGCATCT CATGGTTTTG ATTTTATTTT CCTGACTATT GATGATGTTG	180
10	AGCATCTTTT CAGGTGCTTA GTGGCCATTT GTCCGTCATC TTTGGAGCAG GAACAAATGTC	240
	TTTTCAAGTC CTTTGCCCAT TTTTAAATTG AATTTTTTGT TGTGAGTTG TATATAACAC	300
	CTTTTTTGAA GTAAAAGGTG CACTGTAATA ATCCAGACTG TGTTCCTCCC TTCTCAGGAT	360
15	TCCTACAGGA AGCAAGTAGT AATTGATGGA GAAACCCTGC TCTTGGATAT TCTCGACACA	420
	GCAGGTCAAG AGGAGTACAG TGCAATGAGG GACCAGTACA TGAGGACTGG GGAGGGCTTT	480
20	CTTTGTGTAT TTGCCATAAA TAATACTAAA TCATTGAAG ATATTCACCA TTATAGGTGG	540
	GTTTAAATTG AATATAATAA GCTGACATTA AGGAGTAATT ATAGTTTTTA TTTTTTGAGT	600

- 74 -

CTTTGCTAAT GCCATGCATA TAATATTTAA TAAAAATTTT TAAATAATGT TTATGAGGTA 660
GGTAATATCC CTGTTTTATA AATGAAGTTC TTGGGGGATT AGAGCAGTGG AGTAACTTGC 720
5 TCCAGACTGC ATCGGTAGTG GTGGTGCTGG GATTGAAACC TAGGCCCTGTT TGACTCCACA 780
GCCTTCTGT 789

10 (2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 615 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

20 TCTAGAATTT TTCAGTAGTT TCTGTTTTAC TATTATGATC TACCTGCATA TTAACCTATT 60
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- 75 -

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240 GGAAACTAGG AATTACATTG TTTTCTTTCA GCCAAATTTT ATGACAAAAAG TTGTGGACAG
300 5 GTTTTGAAAG ATATTGTGT TACTAATGAC TGTGCTATAA CTTTTTTTTC TTTCCCAGAG
360 AACAAATTAA AAGAGTTAAG GACTCTGAAG ATGTACCTAT GGTCCCTAGTA GGAATAAAAT
420 GTGATTTGCC TTCTAGAACA GTAGACACAA AACAGGCTCA GGACTTAGCA AGAAGTTATG
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600 15 AATTATGCTA TACTAGGAAA TAAACAATA TTTAGTAAAT GTTTTGTCT CTTGAGAGGG
615 CATTGCTTCT TAATC

20 (2) INFORMATION FOR SEQ ID NO: 5:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 866 base pairs

-76-

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (x1) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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ATTTAAAATA AAACCTCAAGG TTCCAGTTTC TTGACTCACC TTGAGATTC TTTTATGTTT 120
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15 AATTTAAAGC TAACTTCAGT TTAGGCTTTA ATTCAGGAC TAGCAAACTA AAATGGTTGC 300
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20 ACCAGGATTC TAGCCCATAT TTTAAAACTG CATCCTCAGT TTTAATCAA CAGTCTGATG 480
TCTGTTTAAA AAAAAAAAAA TCTCAAGCTC ATAATCTCAA ACTTCTTGCA CATGGCTTTC 540

-77-

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5 TCAGCAAAGA AGAAAAGACT CCTGGCTGTG TGAAAATTAA AAAATGCATT ATAATGTAAT 720
CTGGTAAGTT TAAGTTCAGC ACATTAAATT TGGCAGAAAG CAGATGTCTT TTAAAGGTAA 780
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10 ATAAGAAACT TGTTCACATA ATTAGT 866

(2) INFORMATION FOR SEQ ID NO: 6:

15

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 617 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

-78-

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10 GCATACTAGT ACAAGTGGTA ATTTTGTAC ATTACACTAA ATTATTAGCA TTGTGTTTAG 360
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15 TTAAAATGAC AGTGGAAGTT TTTTTTTCCT CGAAGTGCCA GTATTCCAG AGTTTGGTT 480
TTTGAAC TAG CAATGCCCTGT GAAAAAGAAA CTGAATACCT AAGATTCTG TCTTGGGGTT 540
TTTGGTGCAT GCAGTTGATT ACTTCTTATT TTCTTACCA AGTGTGAATG TTGTTGTGAA 600
20 ACAAAATTAAT GAAGCTT 617

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 574 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..567

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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	1 5 10 15	
20	AGT GCC TTG ACG ATA CAG CTA ATT CAG AAT CAT TTT GTG GAC GAA TAT	96
	Ser Ala Leu Thr Ile Gln Leu Ile Gln Asn His Phe Val Asp Glu Tyr	
	20 25 30	

-80-

144
 GAT CCA ACA ATA GAG GAT TCC TAC AGG AAG CAA GTA GTA ATT GAT GGA
 Asp Pro Thr Ile Glu Asp Ser Tyr Arg Lys Gln Val Val Ile Asp Gly
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192
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 Glu Thr Cys Leu Leu Asp Ile Leu Asp Thr Ala Gly Gln Glu Glu Tyr
 50 55 60

240
 10 AGT GCA ATG AGG GAC CAG TAC ATG AGG ACT GGG GAG GGC TTT CTT TGT
 Ser Ala Met Arg Asp Gln Tyr Met Arg Thr Gly Glu Gly Phe Leu Cys
 65 70 75 80

288
 15 GTA TTT GCC ATA AAT AAT ACT AAA TCA TTT GAA GAT ATT CAC CAT TAT
 Val Phe Ala Ile Asn Asn Thr Lys Ser Phe Glu Asp Ile His His Tyr
 85 90 95

336
 AGA GAA CAA ATT AAA AGA AGT AAG GAC TCT GAA GAT GTA CCT ATG GTC
 Arg Glu Gln Ile Lys Arg Val Lys Asp Ser Glu Asp Val Pro Met Val
 100 105 110

384
 20 CTA GTA GGA AAT AAA TGT GAT TTG CCT TCT AGA ACA GTA GAC ACA AAA
 Leu Val Gly Asn Lys Cys Asp Leu Pro Ser Arg Thr Val Asp Thr Lys
 115 120 125

- 81 -

CAG GCT CAG GAC TTA GCA AGA AGT TAT GGA ATT CCT TTT ATT GAA ACA 432
 Gln Ala Gln Asp Leu Ala Arg Ser Tyr Gly Ile Pro Phe Ile Glu Thr
 130 135 140

 5 TCA GCA AAG ACA AGA CAG AGA GTG GAG GAT GCT TTT TAT ACA TTG GTG 480
 Ser Ala Lys Thr Arg Gln Arg Val Glu Asp Ala Phe Tyr Thr Leu Val
 145 150 155 160

 AGA GAG ATC CGA CAA TAC AGA TTG AAA AAA ATC AGC AAA GAA GAA AAG 528
 10 Arg Glu Ile Arg Gln Tyr Arg Leu Lys Lys Ile Ser Lys Glu Glu Lys
 165 170 175

 ACT CCT GGC TGT GTG AAA ATT AAA AAA TGC ATT ATA ATG TAATCTG 574
 Thr Pro Gly Cys Val Lys Ile Lys Lys Cys Ile Ile Met
 180 185

(2) INFORMATION FOR SEQ ID NO: 8:

- 20 (1) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 189 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

-82-

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

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10  Ser Ala Leu Thr Ile Gln Leu Ile Gln Asn His Phe Val Asp Glu Tyr
    20       25       30

    Asp Pro Thr Ile Glu Asp Ser Tyr Arg Lys Gln Val Val Ile Asp Gly
    35       40       45

15  Glu Thr Cys Leu Leu Asp Ile Leu Asp Thr Ala Gly Gln Glu Glu Tyr
    50       55       60

    Ser Ala Met Arg Asp Gln Tyr Met Arg Thr Gly Glu Gly Phe Leu Cys
    65       70       75       80

    Val Phe Ala Ile Asn Asn Thr Lys Ser Phe Glu Asp Ile His His Tyr
    85       90       95

    Arg Glu Gln Ile Lys Arg Val Lys Asp Ser Glu Asp Val Pro Met Val
    100      105      110

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- 83 -

Leu Val Gly Asn Lys Cys Asp Leu Pro Ser Arg Thr Val Asp Thr Lys
115 120 125

Gln Ala Gln Asp Leu Ala Arg Ser Tyr Gly Ile Pro Phe Ile Glu Thr
5 130 135 140

Ser Ala Lys Thr Arg Gln Arg Val Glu Asp Ala Phe Tyr Thr Leu Val
145 150 155 160

Arg Glu Ile Arg Gln Tyr Arg Leu Lys Lys Ile Ser Lys Glu Glu Lys
10 165 170 175

Thr Pro Gly Cys Val Lys Ile Lys Lys Cys Ile Ile Met
180 185

15

- 84 -

6. CLAIMS:

1. An adenoviral expression vector comprising a promoter functional in eukaryotic cells and a K-ras polynucleotide, wherein said polynucleotide is under transcriptional control of said promoter such that a K-ras transcript synthesized therefrom is antisense.
2. The adenoviral expression vector according to claim 1, further comprising a polyadenylation signal.
3. The adenoviral expression vector according to claim 1, further comprising a selectable marker.
4. The adenoviral expression vector according to claim 1, wherein said polynucleotide consists essentially of exon 2, intron 3 and exon 3.
5. The adenoviral expression vector according to claim 1, wherein said polynucleotide consists essentially of the 1.7 kB Nde I-Sal I fragment of the K-ras protooncogene.
6. The adenoviral expression vector according to claim 1, wherein said expression vector is replication deficient.
7. The adenoviral expression vector according to claim 6, wherein said expression vector lacks at least a portion of the E1 region.

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8. A pharmaceutical composition comprising (i) an adenoviral expression vector comprising a promoter functional in eukaryotic cells and a K-ras polynucleotide, wherein said polynucleotide is under transcriptional control of said promoter such that a K-ras transcript synthesized therefrom is antisense and (ii) a pharmaceutically acceptable buffer, solvent or diluent.

10

9. A method for inhibiting K-ras function in a cell comprising the steps of:

15 (i) providing an adenoviral expression vector comprising a promoter functional in eukaryotic cells and a K-ras polynucleotide, wherein said polynucleotide is under transcriptional control of said promoter such that a K-ras transcript synthesized therefrom is antisense; and

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(ii) contacting said expression vector with said cell.

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10. The method according to claim 9, wherein said cell is a transformed cell and said contacting reverses said transformed phenotype.

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11. The method according to claim 10, wherein said cell is a tumor cell.

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12. The method according to claim 11, wherein said tumor cell is a lung cancer, pancreatic cancer or colon cancer cell.

5

13. The method according to claim 9, wherein said expression vector is packaged in an adenoviral capsid and said contacting comprises infecting said cell.

10

14. A method of treating a mammal with cancer comprising:

(i) providing a pharmaceutical composition comprising (a) an adenoviral expression vector comprising a promoter functional in eukaryotic cells and a K-ras polynucleotide, wherein said polynucleotide is under transcriptional control of said promoter such that a K-ras transcript synthesized therefrom is antisense, and (b) a pharmaceutically acceptable buffer, solvent or diluent; and

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(ii) administering said pharmaceutical composition to said mammal.

25

15. The method according to claim 14, wherein said mammal is a human.

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16. The method according to claim 15, wherein said administering is via intravenous injection.

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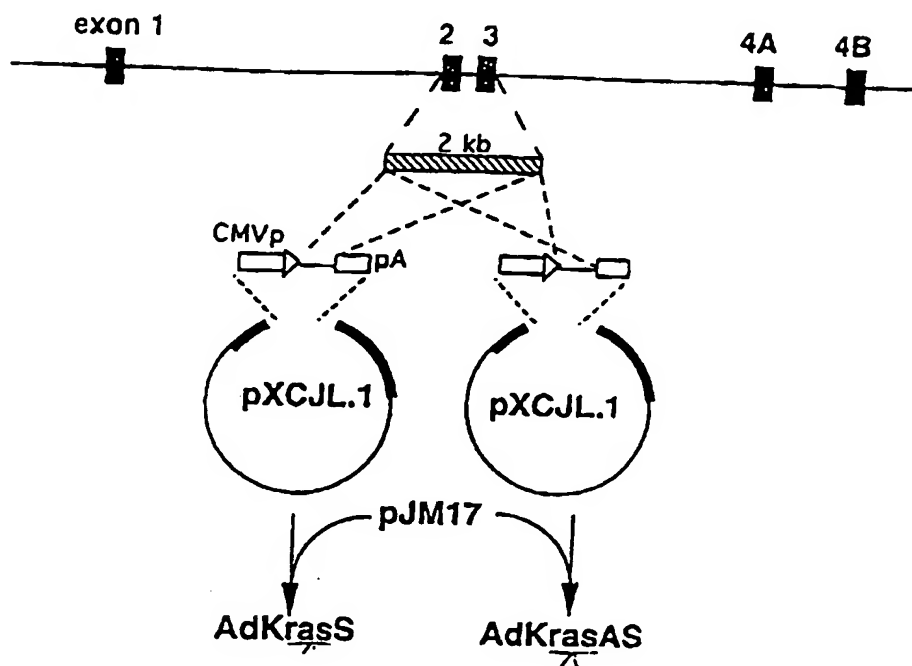
17. The method according to claim 15, wherein said administering is via orthotopic injection.

5 18. The method according to claim 14, wherein said cancer is lung cancer, pancreatic cancer or colon cancer.

10 19. The method according to claim 14, wherein said expression vector is packaged in an adenoviral capsid and said contacting comprises infecting said cell.

15 20. A kit comprising, in suitable container means, an expression vector comprising a promoter functional in eukaryotic cells and a polynucleotide encoding a K-ras antisense transcript, wherein said nucleic acid is under transcriptional control of said promoter, and a pharmaceutically acceptable buffer, solvent or diluent.

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ADENOVIRAL VECTOR CONSTRUCTION

FIG. 1

2 / 2

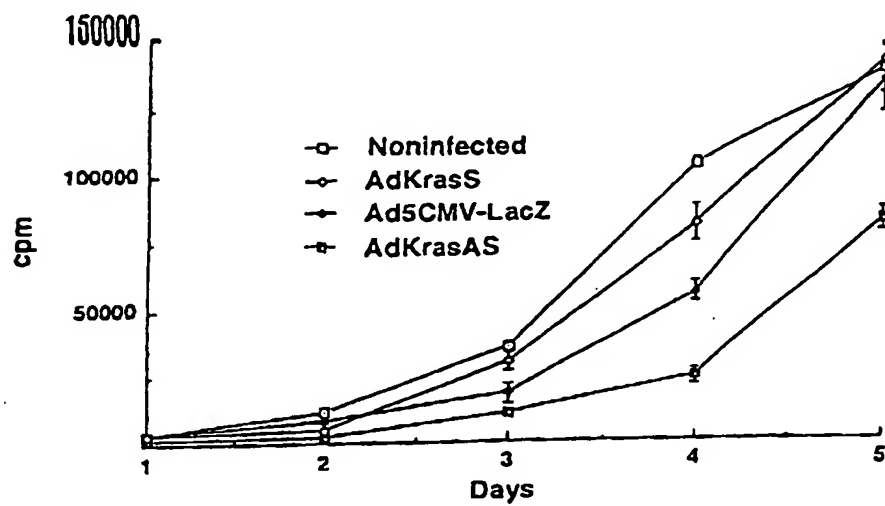
GROWTH CURVE OF TRANSDUCED H460a CELL IN VITRO

FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/17979

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/11 C12N15/86 A61K31/70 //A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 15680 A (UNIV TEXAS) 17 September 1992	1-5, 9-12,14, 15,18,20
Y	see page 6, line 17 - page 10, line 25 see page 21, line 1 - line 12 see claims	1,6-8, 13,16, 17,19
Y	--- WO 95 02697 A (RHONE POULENC RORER SA ;PERRICAUDET MICHEL (FR); VIGNE EMMANUELLE) 26 January 1995 see page 3, line 9 - line 25 see page 6, line 25 - line 30 see examples see claims 1,12,14,28-30 ---	1,6-8, 13,16,19
	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

24 February 1997

Date of mailing of the international search report

04.03.97

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Authorized officer

Andres, S

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/17979

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CANCER RES., (1993) 53/8 (1743-1746), XP002025920 GEORGES R.N. ET AL: "Prevention of orthotopic human lung cancer growth by intratracheal instillation of a retroviral antisense K - ras construct." cited in the application see the whole document ---	17
O,A	PROC ANNU MEET AM ASSOC CANCER RES, (MARCH 1994). VOL. 35, PP. 692-3., XP002025921 ROTH. J. A.: "Gene replacement strategies for therapy and prevention of lung cancer." ---	
O,A	ANTISENSE RES.DEV. 5 (SPRING 95); 109; OP-33 , XP002025922 FUNATO, T. ET AL.: "Effects of tumor growth suppression in cancers by antisense-ras ribozyme" & 1ST INT.ANTISENSE CONF.JAPAN (04 TO 07-12-94), ---	
A	HUMAN GENE THERAPY, vol. 4, 1 January 1993, pages 461-476, XP002004336 RICH, D. ET AL.: "DEVELOPMENT AND ANALYSIS OF RECOMBINANT ADENOVIRUSES FOR GENE THERAPY OF CYSTIC FIBROSIS" cited in the application ---	
P,X	CANCER GENE THERAPY, (1996 SEP-OCT) 3 (5) 296-301., XP000645028 ALEMANY, R. ET AL.: "Growth inhibitory effect of anti- K - ras adenovirus on lung cancer cells." see the whole document -----	1-7, 9-14,20

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/ 17979

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 9-13
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 9-13 (as far as in vivo methods are concerned) and 14-19 are directed to a method of treatment of (diagnostic method practised on) the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No
PCT/US 96/17979

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		NZ 269156 A	26-03-96
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